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Trans-10, cis-12 Conjugated Linoleic Acid Decreases *de novo* Lipid Synthesis in Human Adipocytes

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Abstract

Conjugated linoleic acid (CLA) reduces adiposity in vivo. However, mechanisms mediating these changes are unclear. Therefore, we treated cultures of human adipocytes with trans-10, cis-12 (10,12) CLA, cis-9, trans-11 (9,11) CLA, or other trans fatty acids (FA) and measured indices of lipid metabolism. The lipid-lowering effects of 10,12 CLA were unique, as other trans FA did not reduce TG content to the same extent. Using low levels of [¹⁴C]-CLA isomers, it was shown that both isomers were readily incorporated into acylglycerols and phospholipids, albeit at lower levels than [¹⁴C]-oleic or [¹⁴C]-linoleic acids. When using [¹⁴C]-acetic acid and [¹⁴C]-pyruvic acid as substrates, 30 μM 10,12 CLA, but not 9,11 CLA, decreased *de novo* synthesis of triglyceride (TG), free FA, diacylglycerol, cholesterol esters, cardiolipin, phospholipids, and ceramides within 3–24 h. Treatment with 30 μM 10,12 CLA, but not 9,11 CLA, decreased total cellular lipids within 3 d and the ratio of monounsaturated FA (MUFA) to saturated FA, and increased C18:0 acyl-CoA levels within 24 h. Consistent with these data, stearoyl-CoA desaturase (SCD)-1 mRNA and protein levels were down-regulated by 10,12 CLA within 7–12 h, respectively. The mRNA levels of liver X receptor (LXR)α and sterol regulatory element binding protein (SREBP)-1c, transcription factors that regulate SCD-1, were decreased by 10,12 CLA within 5 h. These data suggest that the isomer-specific decrease in *de novo* lipid synthesis by 10,12 CLA is due, in part, to the rapid repression of lipogenic transcription factors that regulate MUFA synthesis, suggesting an anti-obesity mechanism unique to this trans FA.

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Keywords

conjugated linoleic acid; adipocytes; lipid synthesis; stearoyl-CoA desaturase

Introduction

Conjugated linoleic acid (CLA) consists of a group of positional and geometric fatty acid (FA) isomers of linoleic acid (C18:2; cis-9, cis-12 octadecadienoic acid). Isomers of CLA are found naturally in ruminant meats and dairy products, and in relatively larger quantities in dietary supplements or fortified foods. The two major isomers of CLA, trans-10, cis-12 (10,12) and cis-9, trans-11 (9,11), have been reported to prevent obesity, diabetes, atherosclerosis, or cancer depending on the specific doses, isomers, and models used. There is a great deal of interest in CLA as a weight loss treatment, because of its ability to decrease body weight and body fat mass in many animal studies, and in some human studies (Reviewed in 1 and 2). Of the two major isomers, 10,12 CLA is responsible for its anti-obesity properties (3–7).

Potential mechanisms by which 10,12 CLA prevents obesity include 1) increasing energy expenditure and lipolysis, 2) inducing adipocyte apoptosis, and 3) decreasing adipogenesis or lipogenesis (Reviewed in 2). For example, CLA suppresses preadipocyte differentiation in animal (8–14) and human preadipocytes (5, 16), in part, via attenuating the expression or activity of peroxisome proliferator activated receptor (PPAR) γ , a transcription factor essential for adipogenesis. Indeed, rodents supplemented with 10,12 CLA had decreased expression of PPAR γ and its target genes (14, 17–21). In mature, primary human adipocytes or murine 3T3-L1 adipocytes, 10,12 CLA decreases the expression or activity of PPAR (8, 22), and PPAR target genes and lipid content (15, 16). Other transcription factors involved in adipogenesis (e.g., CCAT/enhancer binding protein α , C/EBP α) or lipogenesis (e.g., sterol regulatory element binding protein 1c, SREBP-1c; liver X receptor α , LXR α) may also be impaired by CLA (5, 14–16, 19, 20). We previously showed that chronic 10,12 CLA treatment of differentiating primary human preadipocytes decreased the mRNA levels of the delta-9 desaturase stearoyl-CoA desaturase (SCD)-1, a SREBP-1c target gene, after 6 d and the monounsaturated fatty acid/saturated fatty acid (MUFA/SFA) ratio after 12 d of treatment, suggesting that down regulation of SCD-1 precedes reductions in the MUFA/SFA ratio (15). However, the effects and kinetics of CLA on regulators of lipid metabolism and *de novo* lipid metabolism in mature primary human adipocytes have not been reported.

Thus, we wanted to understand the specificity and kinetics of these anti-lipogenic actions of CLA isomers in human adipocytes; i.e., does CLA directly attenuate lipogenic transcription factors, which in turn alter lipid metabolism, or does CLA initially alter lipid metabolism, which in turn affects the expression or activity of these transcription factors. To address these questions, we compared the delipidating effects of 10,12 CLA to other trans FA. Furthermore, we examined the kinetics of CLA's incorporation into lipid classes and its impact on *de novo* lipid synthesis and the expression of LXR, SREBP-1c and their target SCD-1 in newly differentiated human adipocytes.

Materials and Methods

Materials

All cell culture ware were purchased from Fisher Scientific (Norcross, GA, USA). Methanolic-HCl (3N) kit was purchased from Supelco (Belafonte, PA, USA), anhydrous methanol 99.8% from Aldrich (Steinheim, Germany), and the GC standard 68A from NU-CHEK-PREP, Inc. (Elysian, MN, USA). Antibodies for SCD-1 (N-20), donkey-anti goat

(SC-2020), and GAPDH (FL-35) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) and the anti-rabbit antibody from Promega (Madison, WI, USA). Western lighting chemiluminescence substrate was purchased from Perkin Elmer Life Science (Boston, MA, USA). One-step reverse transcription-polymerase chain reaction (RT-PCR) kit used in semi-quantitative mRNA analysis was purchased from Qiagen, Inc (Valencia, CA, USA). Immunoblotting buffers, precast gels and gene-specific primers were purchased from Invitrogen (Carlsbad, CA), and ribosomal 18S competitor technology internal standards and DNA-free were purchased from Ambion (Austin, TX). All other reagents and chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise stated. All reagents and chemicals used to isolate lipids were of analytic grade or better.

Sources of FA

Isomers of CLA (+98% pure) were purchased from Matreya (Pleasant Gap, PA). [1-¹⁴C]-9,11 CLA and [1-¹⁴C]-10,12 CLA (specific activity = 53.4 mCi/mmol; radiochemical and isomeric purities >95%) were synthesized by the Service de Chimie Bioorganique et de Marquage, F-91191, Gif sur Yvette, France. They were prepared by stereoselective multistep synthesis involving sequential substitution of 1,2-dichloro-ethene as previously described (23). [¹⁴C]-oleic acid and [¹⁴C]-linoleic acid (specific activities of 50 mCi/mmol), [¹⁴C]-acetate (specific activity of 57 mCi/mmol), and [¹⁴C]-pyruvate (specific activity of 15 mCi/mmol) were purchased from Amersham Biosciences (Buckinghamshire, UK). Unlabeled oleic acid, elaidic acid, vaccenic acid, and trans vaccenic acid were purchased from Sigma-Aldrich.

Methods

Culturing of human primary adipocytes

Abdominal white adipose tissue (WAT) was obtained from non-diabetic females, between the ages of 20–50 years old with a body mass index >30 during abdominoplasty with consent from the Institutional Review Board at the University of North Carolina at Greensboro. Tissue was digested using collagenase and stromal vascular (SV) cells were isolated as previously described (15). Experimental treatment of cultures containing ~50% preadipocytes and ~50% adipocytes occurred on day 6 of differentiation. Each experiment was done in duplicate and repeated at least once using a mixture of cells from 2–3 subjects unless otherwise indicated. These cells were used for data presented in Figures 6 and 7 due to the collaborative nature of this research.

Culturing of human Simpson–Golabi–Behmel Syndrome (SGBS) cells

SGBS is a rare X-linked disorder characterized by pre- and postnatal overgrowth. The cell strain exhibits a high capacity for adipose differentiation, resulting in mature fat cells which are biochemically and functionally similar to human primary adipocytes (24). They offer an advantage over human primary adipocytes, because they can be grown and differentiated in much greater quantities. For these reasons and due to the collaborative nature of this research, they were used in all of the experiments performed with the exception of data shown in Figures 6 and 7. They were generously provided by Dr. Martin Wabitsch. SGBS cells were grown to confluence in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham and supplemented with 10% bovine serum, 33 μM biotin, 17 μM pantothenate, 100 μg/ml streptomycin and 62 μg/ml penicillin. To induce differentiation, SGBS cells were repeatedly washed with PBS buffer and cultured in serum-free medium supplemented with 350 nM insulin, 200 pM triiodothyronine, 1 μM cortisol, 2 μM BRL 49653 (Rosiglitazone), 0.115 mg/ml 1-methyl-3-isobutylxanthine (IBMX), 0.25 mmol/L dexamethasone (DEX) and 0.01 mg/ml human transferrin for 4 d. After 4 d media was removed and replaced with

the differentiation media in the absence of BRL 49653, IBMX, and DEX. All treatments of these cells started on day 6 of differentiation. These cells were used for data in Figures 1–5, 8, and 9.

Preparation of FA

Isomers of CLA, oleic acid, elaidic acid, vaccenic acid and trans vaccenic acid were complexed to FA-free- bovine serum albumin (BSA) (>98%, A7030, Sigma-Aldrich) at a 4:1 molar ratio using 1 mM BSA stocks. All cultures were continuously treated with FAs which were added fresh with each media change (every 3–4 d).

Complexing of [¹⁴C]-FA to BSA

10 nmol of cold FA (e.g. 10,12 CLA) was evaporated under nitrogen gas and 1 μCi of [¹⁴C]-FA added and subsequently evaporated again. 10 μl of a 1.1 mM KOH solution was added and incubated for 10 min at 37°C, centrifuged at 2,000 rpm for 1 min and 90 μl of a 2.5 mM BSA stock was added, the solution was left at room temperature for 2–3 h and then overnight at 4°C and subsequently stored at –20°C.

[¹⁴C]-FA incorporation into lipid classes

Following treatments, cultures were incubated for multiple time points with 1 μCi of [¹⁴C]-9,11 CLA, [¹⁴C]-10,12 CLA, [¹⁴C]-oleic acid, or [¹⁴C]-linoleic acid, harvested, and the lipids extracted using the Bligh and Dyer method (25). Neutral and compound lipids were then separated by high performance thin layer chromatography (HPTLC) on silica gel 60 plates (Merck KGaA, 50 Darmstadt, Germany) and subsequently developed in (hexane:diethylether:acetic acid) (70:30:1 v/v) and chloroform/ethanol/water/triethyl-amine (30:35:7:35, v/v), respectively, incubated overnight on phospho-imager screens, and labelled lipids were detected and quantified using a Typhoon scanner and ImageQuant TL (GE Healthcare, Pittsburgh, PA).

De novo synthesis of neutral and compound lipids using [¹⁴C]-acetate and [¹⁴C]-pyruvate

After treatment, 100 μL of PBS containing 100 nM insulin and 2 μCi [¹⁴C]-acetate or 1 μCi [¹⁴C]-pyruvate were added to each plate and incubated for 240 min (based on results of a preliminary study). In all cultures, the labeling media was removed and the cells were washed in ice-cold Krebs-Ringer buffer, washed once with ice-cold PBS, harvested in 1 ml PBS, pH 7.4, and immediately frozen at –80°C. For neutral lipid detection, lipids were extracted [25], separated by HPTLC in hexane:diethylether:acetic acid (70:30:1 v/v), incubated overnight on phospho-imager screens, the lipid spots were detected and quantified using a Typhoon scanner and ImageQuant TL. For compound lipid detection, lipids were extracted [25], separated on boric acid (2.3%)-treated HPTLC plates in chloroform/ethanol/water/triethylamine (30:35:7:35, v/v), and then processed like the neutral lipid procedure. For ceramide detection, extracted lipids were subjected to a mild alkaline hydrolysis to purify the amide-bound lipids, then separated twice by HPTLC in chloroform:methanol:acetic acid (190:9:1 v/v/v), incubated overnight on phospho-imager screens, and detected as described above. Protein concentrations were determined using the Qubit™ fluorometer (Invitrogen) and the Quant-iT™ protein assay kit as described by the manufacturer. Similar experiments were performed using ¹⁴C-pyruvate. Because the results from the ¹⁴C-acetate and ¹⁴C-pyruvate were similar, only data from the ¹⁴C-acetate are shown.

Gas Chromatography (GC)

For measuring FA profiles, total lipids were saponified, methylated, and analyzed using GC. Briefly, cells were lysed by sonication and the protein concentration was determined. Before

extraction, an internal standard C15:0 (pentadecanoic acid) was added. Total cellular lipids were extracted with chloroform/methanol (1:2 v/v) [25]. Aliquots of the lipid extracts were dried under nitrogen gas and converted to FA methyl esters (FAME) by using anhydrous 99.8% MeOH/Methanolic-HCl (3N) (2:1 v/v), heated at 60°C in oil bath for 20 min and identified by comparison to the 68A standard and vaccinate 18:1(n-7) (Larodan, Malmö, Sweden) using GC. GC was performed on a Chrompack CP 9002, equipped with a CPSIL88 FAME 0.25mm ID capillary column (Chrompack, 51 Middelburg, The Netherlands) and the oven temperature was programmed from 120°C to 220°C, with an increment of 3°C/min. The FA composition of the total lipids was determined (% of total), from which the ratios of 14:1/14:0, 16:1/16:0, and 18:1/18:0 were calculated.

HPLC

Cells were harvested, centrifuged, and the supernatant discarded and cells were stored at -80°C until acyl-CoA extraction. Identification of individual acyl-CoAs was performed using standard acyl-CoA mixtures as we described (26).

RNA isolation and real time qPCR

Total RNA was isolated from the cultures using Tri Reagent (Molecular Research Center, Inc, Cincinnati, OH) according to manufacturer's protocol. RNA was extracted with phenol/1-bromo-3-chloropropane, and precipitated with ethanol, dried, and resuspended in H₂O. Contaminating genomic DNA was removed by treatment with DNase (DNA-free; Ambion). First strand cDNA synthesis and real time quantitative PCR (qPCR) were carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as previously described (15). Primer sets for SCD-1, LXR α , SREBP-1c, PPAR γ , and TATA-binding protein (TBP) are shown in Table 1.

Statistics

Statistical analyses for data in Figures 2, 5, 6, 7B, and 8A, and 9B were performed testing the main effects of treatment time and FA type and the interaction of the two (time x FA) using two-way ANOVA (JMP Version 6.03, SAS Institute; Cary, NC). Statistical analyses for data in Figures 1B–D, 2, and 8B,C were conducted using a one-way ANOVA. Student's t tests were used to compute individual pairwise comparisons of least square means ($P < 0.05$). Data are expressed as the means \pm S.E.M.

Results

Trans-10, cis-12 CLA, but not other trans FA, delipidates adipocytes

Previous studies have reported that trans FA such as elaidic acid (trans-9, C18:1) increase lipolysis (27) and inflammation (28) and reduce PPAR γ expression (29) similar to that of 10,12 CLA. To examine if some or all of the effects of 10,12 CLA are due to the positioning of the trans carbon-carbon double bond in CLA, we treated newly differentiated cultures of SGBS cells with 30 μ M elaidic acid (trans 9, C18:1), trans vaccenic acid (trans 11, C18:1), or 10,12 CLA. Cultures treated with 30 μ M of oleic acid (cis 9, C18:1) or vaccenic acid (cis 11, C18:1) were included as controls for elaidic and trans vaccenic acid, respectively. Cultures treated with 10,12 CLA for 9 d appeared to contain less lipid (Fig. 1A), and had one of the lowest levels of TG compared to the other cultures (Fig. 1B), indicating that these cells were delipidated, as we previously reported for primary human *in vitro*-differentiated adipocytes (16, 22). Furthermore, 10,12 CLA decreased the C16:1/C16:0 (Fig. 1C) and C18:1/C18:0 ratios, (Fig. 1D), although only the C16:1/C16:0 ratio was significantly different than the other FAs. Taken together, these data suggest that the position of the trans

carbon-carbon bond in 10,12 CLA provides a unique mechanism for reducing the TG content and the MUFA/SFA ratio in human adipocytes compared to other trans FA.

[¹⁴C]-10,12 CLA and [¹⁴C]-9,11 CLA incorporate equally into lipids

Although we previously demonstrated that CLA incorporates into neutral and phospholipids during human preadipocyte differentiation (15), the kinetics of CLA's incorporation into specific lipid classes in mature adipocytes has not been determined. Thus, newly differentiated SGBS adipocytes were incubated on day 6 of differentiation with low levels of [¹⁴C]-9,11 CLA and [¹⁴C]-10,12 CLA, and their incorporation into specific lipid classes was determined by separating the lipid classes using HPTLC and determining their rate of incorporation into neutral and compound lipids. Cultures were also incubated with low levels of [¹⁴C]-oleic and [¹⁴C]-linoleic acid as FA controls. Both [¹⁴C]-isomers of CLA incorporated into phosphatidylcholine, cardiolipin, and TG at similar rates and amounts (Fig. 2). The rates of [¹⁴C]-oleic and [¹⁴C]-linoleic acid incorporation followed relatively similar patterns (Fig. 2), although they incorporated to a greater extent than the CLA isomers. These differences in incorporation into phosphatidylcholine, cardiolipin, and TG became apparent after 0.3, 3, and 10 h, respectively. Incorporation of [¹⁴C]-isomers of CLA into other phospholipid classes was not sufficient enough above background to be detected (data not shown). Thus, when given at low levels, CLA isomers readily incorporate into lipid classes equally, albeit at lower levels than oleic and linoleic acids.

Trans-10, cis-12 CLA decreases *de novo* lipid synthesis

To investigate the influence of 10,12 CLA on *de novo* lipid synthesis, cultures of newly differentiated SGBS cells were treated on day 6 of differentiation with BSA, 30 μM 9,11 CLA, or 30 μM 10,12 CLA for 3, 24, or 72 h. Subsequently, cultures were incubated for 4 h, based on the results of a pilot study (data not shown), with either [¹⁴C]-acetate or [¹⁴C]-pyruvate as substrates. Because the results from both labelled substrates were relatively similar, only data from the [¹⁴C]-acetate studies are shown here. Treatment for 3–24 h with 30 μM 10,12 CLA, but not 9,11 CLA, decreased *de novo* synthesis of neutral lipids (Fig. 3) including cholesterol esters, TG, free FA, cholesterol, and diacylglycerols, and compound lipids (Fig. 4) including cardiolipin, phosphatidic acid, and phospholipids compared to the BSA vehicle. Quantification and statistical analyses of some of these lipid spots on the HPTLC plates are shown in Figure 5. Similarly, *de novo* synthesis of non-hydroxy ceramide (NS) and ceramide-containing sphingosine (EOS), ceramide-containing lipids made from SFA, were lower in cultures treated with 10,12 CLA for 3, 24, and 72 h compared to 9,11 CLA and BSA (Fig. 6). Collectively, these data demonstrate that 10,12 CLA markedly reduces *de novo* synthesis of neutral, phosphoglycerol, and complex lipids within 3–24 h in an isomer-specific manner.

Trans-10, cis-12 CLA decreases the lipid content and MUFA/SFA ratio

Although we previously demonstrated that chronic treatment with 10,12 CLA reduced the lipid content and MUFA/SFA ratio of differentiating human preadipocytes (15) and in mature human adipocytes (Fig. 1), we did not know the kinetics of this suppression. Therefore, newly differentiated primary adipocytes were treated on day 6 of differentiation with BSA (vehicle), 9,11 CLA (isomer control), or 10,12 CLA and harvested after 3 h, 1 d, 3 d, or 9 d of treatment. Trans-10, cis-12 CLA reduced the TG levels (Fig. 7A) after 3 d and the MUFA/SFA ratio (Fig. 7B) after 1 d of treatment compared to 9,11 CLA and the BSA vehicle. Whereas 10,12 CLA decreased total acyl-CoAs (Fig. 8A) after 24–72 h of treatment, it increased the percentage of C18:0 acyl-CoA (Fig. 8B,C), which has been implicated in causing insulin resistance (30).

Trans-10, cis-12 CLA decreases SCD-1 protein and mRNA levels

Given the important role that SCD-1 plays in desaturating long chain SFA into MUFA needed for lipid storage and metabolism, we examined the impact of CLA isomers on SCD-1 mRNA and protein levels in mature adipocytes. SGBS adipocytes were treated on day 6 of differentiation with BSA, 30 μ M 9,11 CLA, or 30 μ M 10,12 CLA, and harvested after 6–72 h for protein or 3–36 h for mRNA expression. Trans-10, cis-12 CLA reduced the protein (Fig. 9A) and mRNA levels (Fig. 9B) of SCD-1 after 12 and 7 h of treatment, respectively, compared to 9,11 CLA or BSA vehicle. The mRNA levels of two transcription factors that are essential for the transcriptional regulation of SCD-1, SREBP-1c and LXR, were decreased after 5 h of treatment with 10,12 CLA compared to BSA vehicle and 9,11 CLA (Fig. 9B). The mRNA levels of PPAR γ , another important regulator of lipogenesis in adipocytes, were similarly decreased by 10,12 CLA. Taken together, these data support the hypothesis that 10,12 CLA decreases lipid metabolism not only by rapidly suppressing the mRNA levels of PPAR γ , but also LXR α and SREBP-1c and their target gene SCD-1, a desaturase required for the synthesis of MUFA essential for neutral and compound lipid synthesis.

Discussion

The purpose of this study was to determine the extent to which CLA isomers impacted *de novo* lipid synthesis in human adipocytes. We found that the lipid-lowering effect of 10,12 CLA does not appear to be due to a “structural trans effect”, because several other trans FA did not delipidate adipocytes or alter the MUFA/SFA ratio to the extent of 10,12 CLA. Alternatively, these effects may be due to the position of the trans double bond in the C18:2 structure of CLA. Consistent with these data, we found that 10,12 CLA, but not 9,11 CLA, suppressed *de novo* lipid synthesis within 3–24 h of treatment. This suppression does not appear to be due to impaired incorporation of 10,12 CLA, as both CLA isomers equally incorporate into lipids, albeit at lower levels than other unsaturated FA. Notably, we demonstrated that 10,12 CLA decreases the MUFA/SFA ratio within 24 h and the mRNA and protein levels of SCD-1, a delta-9 desaturase essential for MUFA synthesis, metabolism, and TG storage, within 7 h in an isomer-specific manner (see Table 2 for the kinetics of these effects). Furthermore, the mRNA levels of LXR α and SREBP-1c were similarly decreased by 10,12 CLA within 5 h. Taken together, these data suggest that 10,12 CLA rapidly reduces SCD-1 abundance and activity in human adipocytes as it does in murine adipocytes (12, 31–33), possibly by reducing the levels of LXR α , SREBP-1c, and PPAR γ that regulate its expression or activity. The rapid 10,12 CLA-mediated reduction in the expression of these lipogenic transcription factors and SCD-1 is likely responsible, in part, for the reduction in MUFA, which could impair neutral and compound lipid synthesis, metabolism, or storage.

These and previously published data suggest that 10,12 CLA lowers the lipid content of adipocytes by rapidly 1) decreasing SCD-1 activity, thereby reducing the MUFA needed for neutral and compound lipid synthesis (reviewed in 34), 2) decreasing PPAR γ activity, thereby reducing the expression of adipogenic and lipogenic proteins needed for lipid biosynthesis (reviewed in 2), or 3) increasing inflammatory lipid metabolites or signals that antagonize glucose and FA uptake and subsequent metabolism (reviewed in 2).

Stearoyl-CoA desaturase-1 is a central lipogenic enzyme (reviewed in 34), and Ntambi first proposed that inhibition of SCD-1 by 10,12 CLA in mice (12, 31, 32) and in humans (33) is key to the anti-lipogenic effects of 10,12 CLA. Stearoyl-CoA desaturase-1 and diacylglycerol transferase-2 (DGAT2) have been shown to co-localize in the ER and be important for TG synthesis (35). Dietary and endogenous palmitate (C16:0) and stearate (C18:0) are desaturated by SCD-1 and channeled to DGAT2 for the final step in TG

synthesis in the ER. This close association between SCD-1 and DGAT2 enhance the efficiency of TG synthesis (35). However, 10,12 CLA's reduction in body fat mass was similar in SCD-1 knockout and wild type mice, suggesting that CLA's antiobesity properties are independent of SCD-1 in mice (36).

We previously demonstrated that long-term treatment (i.e., 12 d) of differentiating primary human preadipocytes with 10,12 CLA decreased the MUFA/SFA ratio and the mRNA levels of SCD-1 (15). Reductions in SCD-1 mRNA were detected after 6 d of treatment in differentiating human preadipocytes (15). We found in the current study that 10,12 CLA reduced the mRNA levels of SCD-1, LXR α , and SREBP-1c within 5–7 h (Fig. 9B) in mature human adipocytes. Similarly, 10,12 CLA decreased the mRNA expression of SREBP-1c in pig preadipocytes (37). Conversely, neither 9,11 CLA nor 10,12 CLA affected SREBP-1a or SREBP-1c mRNA levels or the precursor protein in a bovine mammary cell line, whereas the abundance of the mature fragment of SREBP-1a and SREBP-1c protein was significantly reduced by 10,12 CLA, but not 9,11 CLA (38). Moreover, 10,12 CLA reduced the mRNA expression for critical genes in the lipid synthesis pathway, whereas 9,11 CLA had no effect. Collectively, these data suggest that 10,12 CLA might also affect the SREBP-1 pathway at the level of proteolytic cleavage rather than by affecting the availability of the precursor protein.

Alternatively, 10,12 CLA could reduce lipogenesis by inhibiting transporters of glucose (i.e., GLUT4) or FA (i.e., aP2) or esterification enzymes regulated by PPAR γ [4, 15, 16, 22, 39], thereby limiting the *de novo* synthesis of SFA for desaturation by SCD-1. Less SFA substrate for SCD-1 would limit its activity (reviewed in 34). In agreement with this hypothesis, 10,12 CLA reduced PPAR γ mRNA levels within 5 h (Fig. 9B, which could contribute to reduced PPAR γ activity. Consistent with these data, we have previously shown that 10,12 CLA reduces PPAR γ mRNA [15, 16], protein (22, 39–41), or activity (22, 40) in human adipocytes, and co-supplementation with the PPAR γ agonist Rosiglitazone attenuates this effect along with preventing insulin resistance and delipidation by 10,12 CLA (22). *In vivo*, Belury's group has shown that Rosiglitazone attenuates many of the adverse effects of CLA treatment including insulin resistance, lipodystrophy, or adiponectin depletion (21, 42).

We speculated that 10,12 CLA produces lipid-borne metabolites like ceramide [reviewed in 43] that antagonize PPAR γ and insulin sensitivity, because increased PPAR γ activity is antagonized by pro-inflammatory mediators like NF κ B (44, 45), and our previous finding demonstrating that NF κ B inhibitors or siRNA targeting p65 attenuates 10,12 CLA's suppression of PPAR γ and insulin resistance (39). However, 10,12 CLA decreased overall *de novo* synthesis of several lipid classes including ceramides, TG, and cholesterol esters. Therefore, although 10,12 CLA decreased the ratio of MUFA/SFA, it did not increase the levels of ceramide, a SFA-derived inflammatory lipid that causes insulin resistance (reviewed in 43). Consistent with these data, we found that treatment of human adipocytes with ceramide inhibitors (i.e., myriocin, fumonisin B1) did not attenuate 10,12 CLA-mediated induction of inflammatory genes or suppression of adipogenic/lipogenic genes (data not shown). Thus, ceramide does not appear to play a role in 10,12 CLA-mediated inflammation or insulin resistance. In addition, 10,12 CLA reduced total acyl-CoA levels and increased the amount of C18:0 acyl-CoA, which has been implicated in insulin resistance due to impaired oxidation (30).

Based on our previous results and results from this work we suggest that, because of its unique structure, 10,12 CLA or its lipid-borne metabolites initially (e.g., within 5–7 h) alters the activity of lipogenic transcription factors and their respective downstream targets. Our previous results indicate that this could occur through activation of inflammatory signals

such as NF κ B [39, 41] that antagonize LXR α , PPAR γ , and possibly also SREBP-1. This leads to decreased activity of lipogenic enzymes essential for FA desaturation and incorporation into neutral and compound lipids within 3–24 h. These 10,12 CLA-mediated changes ultimately reduce total cellular lipids within 72 h. Alternatively, 10,12 CLA could also directly affect the activity of desaturases and lipogenic enzymes; however, the kinetics of our data summarized in Table 2 are consistent 10,12 CLA acting primarily by attenuating the activity of lipogenic transcription factors and their targets, which in turn decrease *de novo* lipid metabolism and lipid accumulation, and not vice-versa.

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Abbreviations

ACBP	acyl-CoA binding protein
aP2/FABP4	adipocyte fatty acid binding protein 4
BCA	bicinchoninic acid
BCP	1-bromo-3-chloropropane
BSA	bovine serum albumin
CLA	conjugated linoleic acid
DMEM	Dulbeco's modified eagles medium
DMSO	dimethyl sulfoxide
EA	elaidic acid
EOS	ceramide-containing sphingosine
FA	fatty acids
FBS	fetal bovine serum
GC	gas chromatography
GPDH	glycerol-3-phosphate dehydrogenase
HBSS	Hanks balanced salt solution
HEPES	n[2-hydroxyethyl]piperazine-n'-[2-ethansulfonic acid]
HPTLC	high performance thin layer chromatography
LXR	liver X receptor
MUFA	monounsaturated fatty acid
NS	non-hydroxy ceramide

OA	oleic acid
ORO	oil red O
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
PVDF	polyvinylidene difluoride
RT-PCR	reverse transcription- polymerase chain reaction
RXR	retinoid X receptor
SCD-1	stearoyl-CoA desaturase-1
SDS	sodium dodecyl sulfate
SFA	saturated fatty acid
SGBS	Simpson-Golabi-Behmel Syndrome
SREBP-1C	sterol regulatory element binding protein
SV	stromal vascular
TBP	TATA binding protein
TLC	thin layer chromatography
TG	triglyceride
TVA	trans vaccenic acid
VA	vaccenic acid
WAT	white adipose tissue

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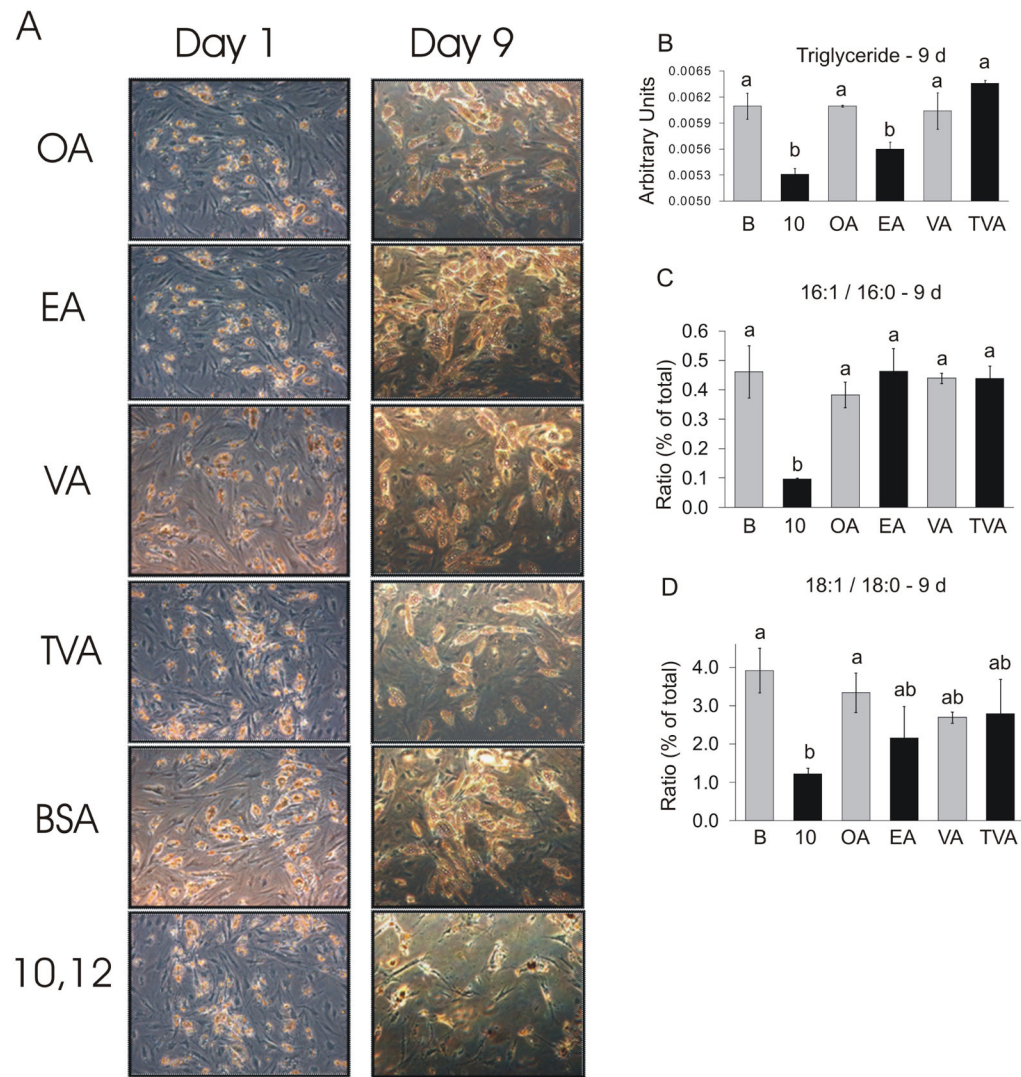


Figure 1. Chronic treatment with trans-10, cis-12 CLA, but not other trans FA, decreases TG content and the MUFA/SFA ratio

Cultures of newly differentiated SGBS adipocytes were treated on day 6 of differentiation for 9 d with 30 μ M oleic acid (OA; cis 9, C18:1), elaidic acid (EA; trans 9, C18:1), vaccenic acid (VA; cis 11, C18:1), trans vaccenic acid (TVA; trans 11, C18:1), BSA vehicle, or 10,12 CLA. A: On Day 0 and Day 9, cultures were visualized using a Leica DM IRBE inverted light microscope with a 10x objective. The pictures are representative of two independent experiments. Left panel, day 0; Right panel, day 9. B: Cultures were harvested and the lipids extracted (25) and spotted onto HPTLC plates and separated in hexane:diethylether: acetic acid (70:30:1 v/v). The separated spots were visualized by spraying the plate with a 3% copper-acetate/15% phosphoric acid solution and charred in a heating cabinet at 100 $^{\circ}$ C for 10–20 min and the quantification of the lipids was based on the intensities of the charred lipids (arbitrary units) using version 4.0.3 of Scion Image. Means (\pm SEM, n=2) not sharing a common superscript are significantly ($P < 0.05$). C,D: The FA composition of the total lipids was determined (% of total), from which the ratios of 16:1/16:0, and 18:1/18:0 were calculated. Means (\pm SEM, n=2) not sharing a common superscript are significantly ($P < 0.05$). Data are all panels are representative of two independent experiments.

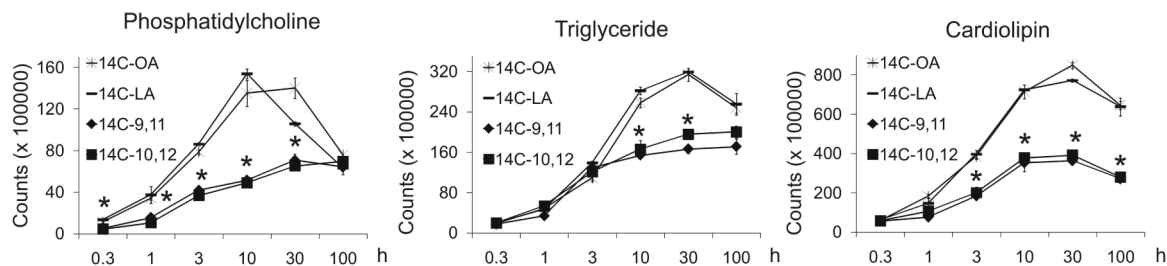


Figure 2. $[^{14}\text{C}]$ -10,12 CLA and $[^{14}\text{C}]$ -9,11 CLA incorporate equally into neutral and compound lipids

Cultures of newly differentiated SGBS adipocytes were treated on day 6 of differentiation with $1 \mu\text{Ci}$ of $[^{14}\text{C}]$ -9,11 CLA, $[^{14}\text{C}]$ -10,12 CLA, $[^{14}\text{C}]$ -9,11 oleic acid (OA), or $[^{14}\text{C}]$ -9,11 linoleic acid (LA) for 20 min, 1 h, 3 h, 10 h, 30 h, or 100 h. Cultures were then harvested and the lipids were extracted (25). Neutral and compound lipids were separated by HPTLC in hexane:diethylether:acetic acid (70:30:1, v/v) and chloroform/ethanol/water/triethylamine (30:35:7:35, v/v), respectively, incubated overnight on phospho-imager screens, and the spots for phosphatidylcholine, cardiolipin, and TG detected and quantified using a Typhoon scanner and ImageQuant TL, respectively. CLA means (\pm SEM, $n=2$) having an asterisks (*) are significantly ($P < 0.05$) different from linoleic and oleic acid for a given time point. Data shown are representative of two individual experiments.

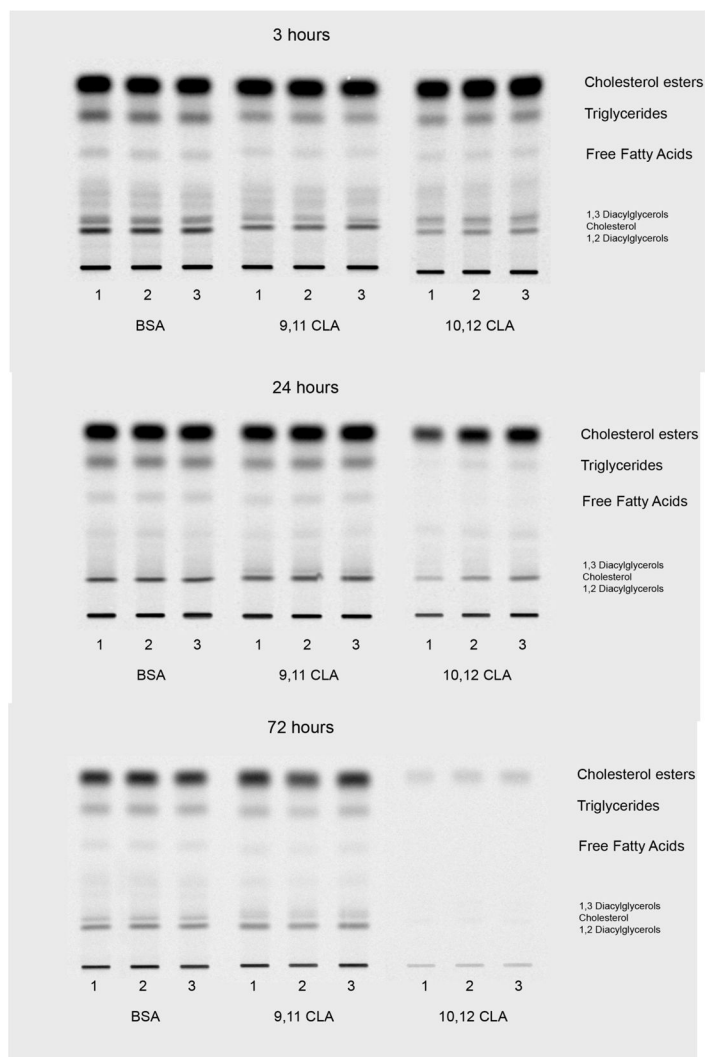


Figure 3. Trans-10, cis-12 CLA decreases *de novo* synthesis of neutral lipids

Cultures of newly differentiated SGBS adipocytes were treated on day 6 of differentiation with BSA, 30 μ M 9,11 CLA, or 30 μ M 10,12 CLA for 3, 24 or 72 h. Cultures were then incubated with 2 uCi of [14 C]-acetate for 240 min and harvested. Lipids were extracted [25], separated by HPTLC in hexane:diethylether:acetic acid (70:30:1 v/v), incubated overnight on phospho-imager screens, the lipid spots were detected. The first three lanes for each treatment represent three different cell culture plates (n=3), and are representative of two independent experiments.

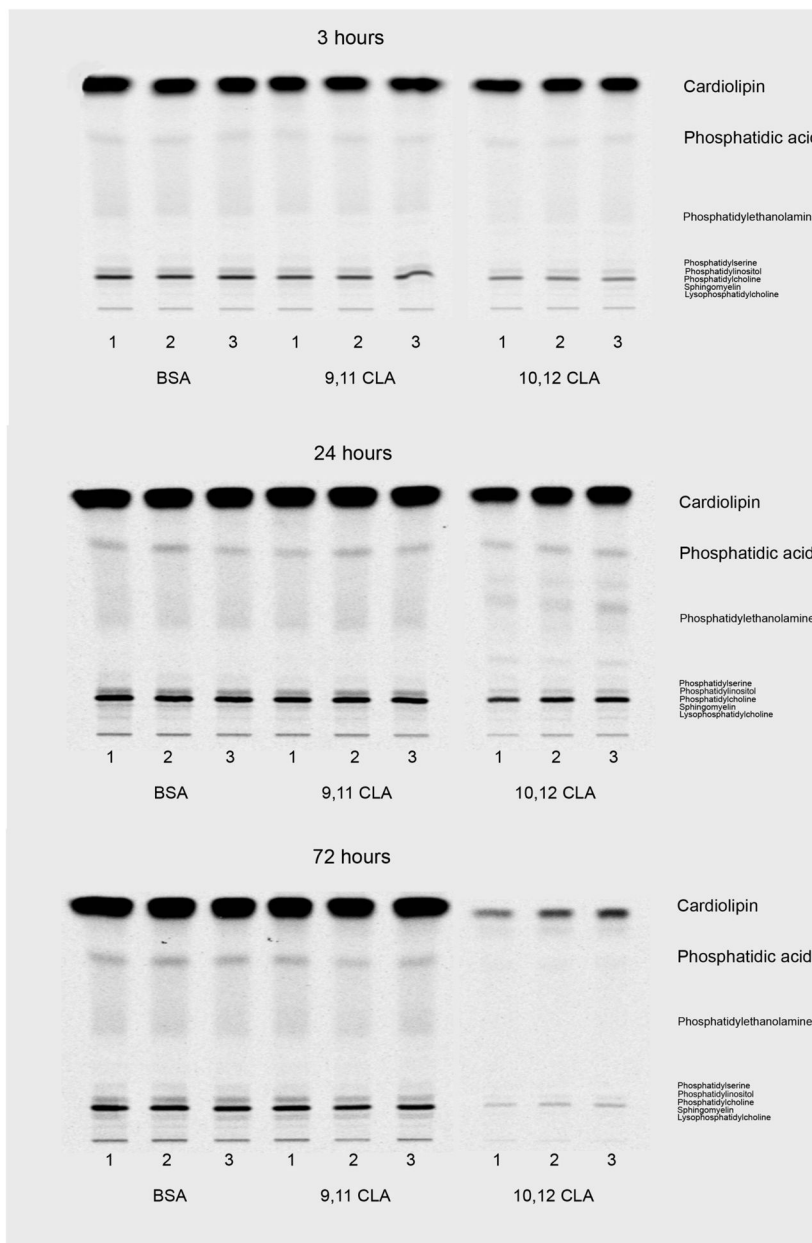


Figure 4. Trans-10, cis-12 CLA decreases *de novo* synthesis of compound lipids

Cultures of newly differentiated SGBS adipocytes were treated on day 6 of differentiation with vehicle (BSA), 30 μ M 9,11 CLA, or 30 μ M 10,12 CLA for 3, 24 or 72 h. Cultures were then incubated with 2 uCi of [14 C]-acetate for 4 h and harvested. Lipids were extracted (25), separated on 2.3% boric acid-treated HPTLC plates in chloroform/ethanol/water/triethylamine (30:35:7:35, v/v), incubated overnight on phospho-imager screens, and the lipid spots detected. The first three lanes for each treatment represent three different cell culture plates (n=3), and are representative of two independent experiments.

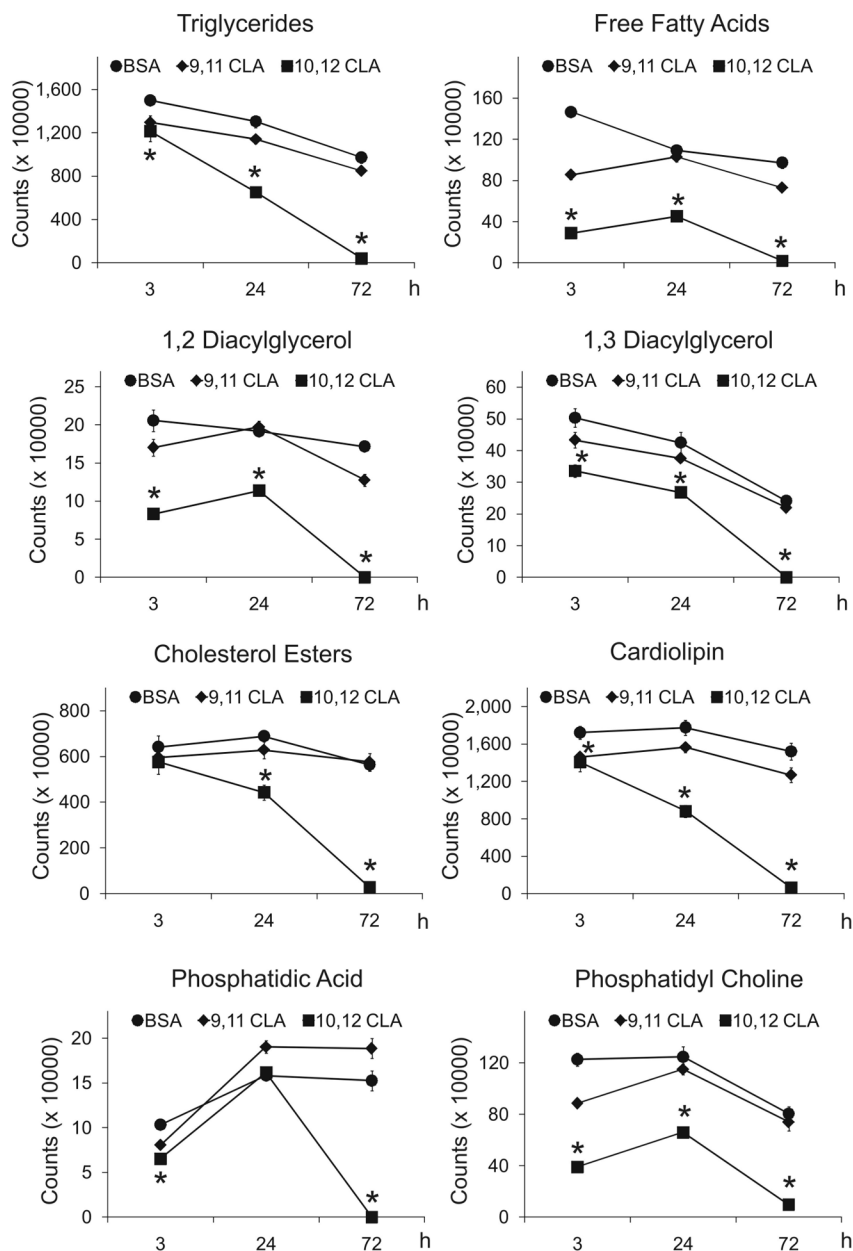


Figure 5. Quantification of *de novo* synthesis of neutral and compound lipids

The intensities of the HPTLC bands from [^{14}C]-acetate incorporation into specific lipid spots in Figures 3 and 4 were quantified using a Typhoon scanner and ImageQuant TL and subjected to statistical analyses. 10,12 CLA means (\pm SEM, n=3) having an asterisks (*) are significantly ($P < 0.05$) different than the BSA vehicle within a given time point. Data are representative of two independent experiments

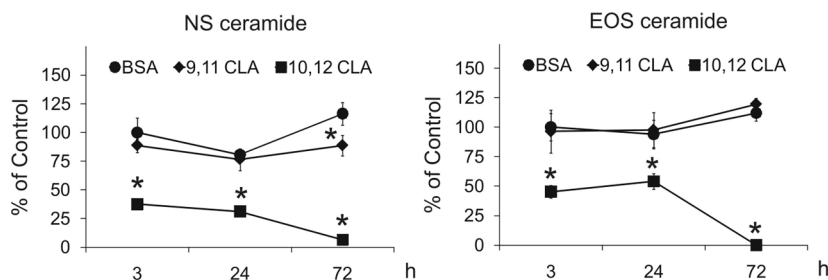


Figure 6. Trans-10, cis-12 CLA decreases *de novo* synthesis of ceramides

Cultures of newly differentiated human adipocytes were treated on day 6 of differentiation with BSA vehicle, 30 μ M 9,11 CLA, or 30 μ M 10,12 CLA for 3, 24, or 72 h. Cultures were then incubated with 2 μ Ci of [14 C]-acetate for 4 h and harvested. Lipids were extracted (25), subjected to a mild alkaline hydrolysis to purify the amide-bound lipids, then separated twice by HPTLC in chloroform:methanol:acetic acid (190:9:1, v/v/v), incubated overnight on phospho-imager screens, and the lipid spots detected and quantified using a Typhoon scanner and ImageQuant TL. Means (\pm SEM, n=3) having an asterisks (*) are significantly ($P < 0.05$) different than the BSA vehicle within a given time point. Data are representative of two independent experiments. Abbreviations used: Cer (NS), Non-hydroxy FA ceramide; Cer (EOS), Ceramide-containing sphingosine.

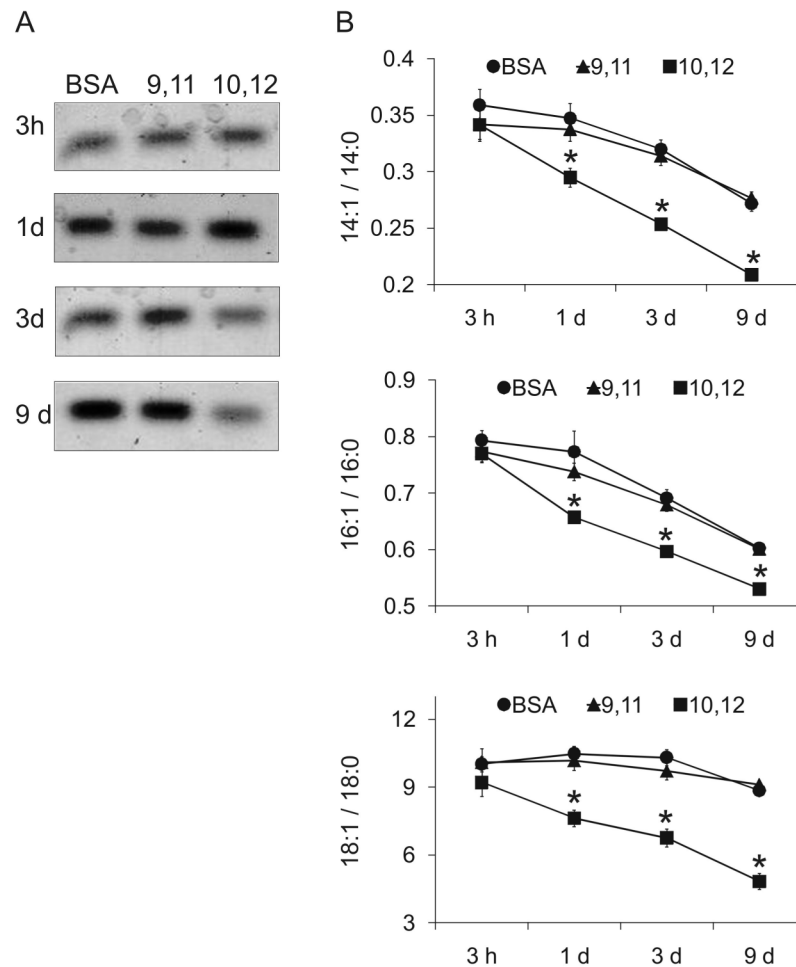


Figure 7. Acute treatment with trans-10, cis-12 CLA decreases the TG content and the MUFA/SFA ratio

Cultures of newly differentiated, primary human adipocytes were treated on day 6 of differentiation with BSA vehicle, 30 μ M 9,11 CLA, or 30 μ M 10,12 CLA for 3 h, 1 d, 3 d, or 9 d, and then total lipids were extracted (25). A: Lipids were separated by HPTLC using a neutral lipid separation system consisting of hexane:diethylether:acetic acid (70:30:1). Data shown are representative of three individual experiments. B: Total lipids were saponified, methylated, and analyzed using GC. The FA composition of the total lipids was determined (% of total), from which the ratios of 14:1/14:0, 16:1/16:0, and 18:1/18:0 were calculated. 10,12 CLA means (\pm SEM, n=8) having an asterisks (*) are significantly ($P<0.05$) different than 9,11CLA and BSA vehicle within a time point.

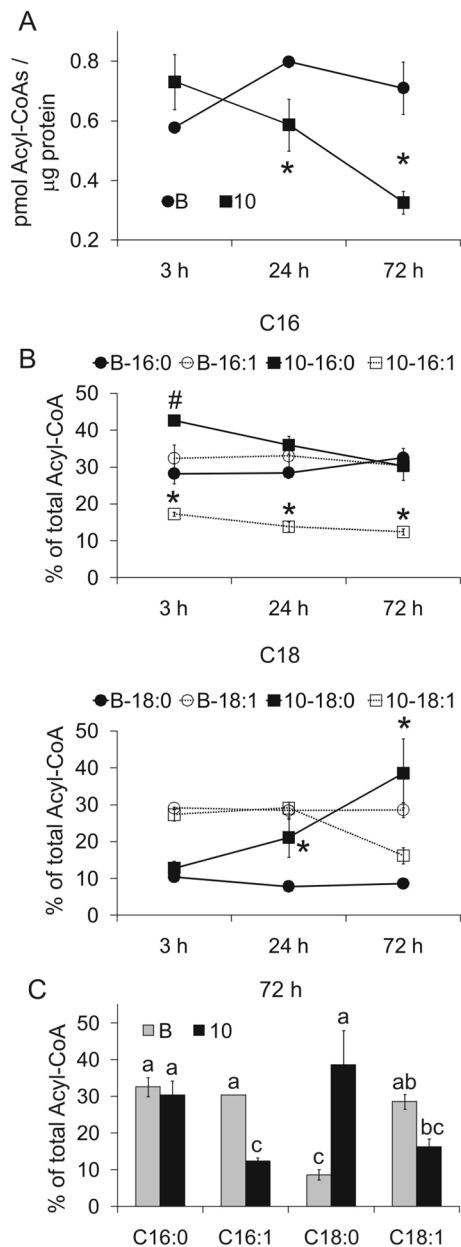


Figure 8. Trans-10, cis-12 CLA changes the rates of desaturation in acyl-CoAs

Cultures of newly differentiated SGBS cells were treated on day 6 of differentiation with BSA vehicle (B) or 30 μ M 10,12 CLA (10) for 3, 24, or 72 h. Cultures were then harvested and the acyl-CoAs were extracted and subsequently derivatized to etheno-acyl-CoAs, which were separated and detected by HPLC using a fluorescence detector. After being normalized to equal protein levels, the total amount of acyl-CoAs (panel A) and C16 and C18 acyl-CoAs (panel B) were calculated using the molecular weight of each individual FA + 767.5 (weight of CoA), compared to the C17:0-CoA internal standard. A: Means (\pm SEM, n=3) having an asterisks (*) are significantly ($P < 0.05$) different than the BSA vehicle within a given time point. B: Means (\pm SEM, n=2) having an asterisks (*) or a pound sign (#) within a FA type (i.e., MUFA is an *; SFA is a #) are significantly different ($P > 0.05$) from one another within a given time point. For panel C, means (\pm SEM, n=2) not sharing a common

superscript are significantly ($P < 0.05$). Data in all panels are representative of two independent experiments.

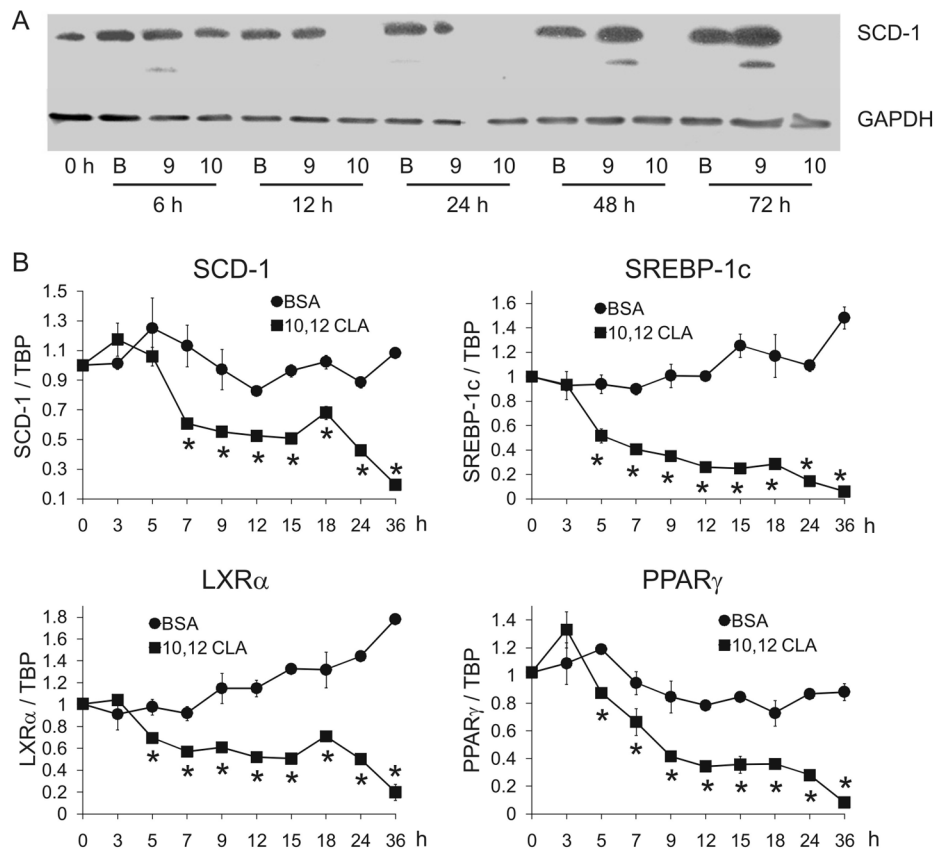


Figure 9. 10, 12 CLA decreases SCD-1 protein and mRNA levels

Cultures of newly differentiated SGBS adipocytes were treated on day 6 of differentiation with BSA vehicle, 30 μ M 9,11 CLA, or 30 μ M 10,12 CLA over time. A: Cultures were treated with BSA, 9,11 CLA or 10,12 CLA for 6, 12, 24, 48, or 72 h. Cells were then harvested and SCD-1 protein and GAPDH levels were determined by immunoblotting. B: Cultures were treated for 3, 5, 7, 9, 12, 15, 18, 24, or 36 h with either a BSA vehicle or 10,12 CLA. Subsequently, cultures were harvested to determine the mRNA levels of SCD1, SREBP-1c, LXR α , PPAR γ , and TATA-binding protein (TBP, load control) by qPCR. The expression level of a given gene was calculated after normalization to TBP expression, and expressed relative to Day 0 (confluent, noninduced) controls. Means (\pm SEM, n=2) having an asterisks (*) are significantly ($P < 0.05$) different than the BSA vehicle within a given time point. Data shown in panels A and B are representative of two individual experiments.

Table 1

Primers used

Target gene	5'-primer	3'-primer
LXR α	TGAAGCGTCAAGAAGAGGAG	ATCAGTGAAGGAATCGCTTTCTG
PPAR γ 2	AGCAAACCCCTATTCCATGCT	GGTGGTCACGAGCCCATTC
SCD-1	CCAACACAATGGCATTCCAG	GGTGGTCACGAGCCCATTC
SREBP-1C	GGAGGGGTAGGGCCAACGGCCT	CATGTCTTCGAAAGTGCAATCC
TBP	GCCCGAAACGCCGAATAT	CCTCATGATTACCGCAGCAAA

LXR α , Liver X-activated receptor; PPAR γ 2, Peroxisome Proliferator Activated Receptor γ 2; SCD-, Stearoyl-CoA desaturase-1; SREBP, Sterol Regulatory Element Binding Protein; TBP, TATA binding protein

Kinetics of 10,12 CLA-mediated suppression of transcription factors or enzymes that regulate lipogenesis and lipid metabolism in human adipocytes.

Table 2

↓Regulators of Lipogenesis by CLA	↓Lipid Metabolism by CLA				
	De novo synthesis			MUFA/SFA Ratio	Total Lipids
5-12 h	3 h	24 h	72 h	24 h	72 h
LXRα	TG	CE	PA	C14:1/C14:0	Total lipids
SREBP-1c	DAG			C16:1/C16:0	
PPARγ	FFA			C18:1/C18:0	
SCD-1	PC				
	CL				
	CER				

Abbreviations used: LXRα, liver X receptor α; SREBP-1c, sterol regulatory element binding protein-1c; PPARγ, peroxisome proliferator activated receptor γ; SCD-1, stearoyl CoA desaturase-1; TG, triglyceride; DAG, diacylglycerol; FFA, free fatty acid; PC, phosphatidylcholine; CL, cardiolipin; CER, ceramide; CE, cholesterol ester; PA, phosphatidic acid; TL, total lipids.